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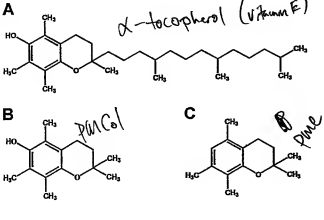
(54) Title: CHROMAN-DERIVED ANTI-ANDROGENS FOR TREATMENT OF ANDROGEN-MEDIATED DISORDERS



(57) Abstract: Methods for the prevention and/or alleviation of androgen-mediated disorders treatable by administering a chroman-derived anti-androgen compound are provided by the present invention. The invention further provides pharmaceutical and nutraceutical compositions containing chroman-derived anti-androgen compounds useful in the prevention and/or alleviation of androgen-mediated disorders, particularly prostate cancer.

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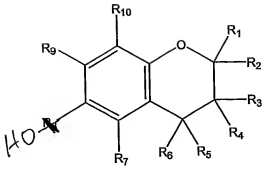
It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the chemicals, cell lines, vectors, animals, instruments, statistical analysis and methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

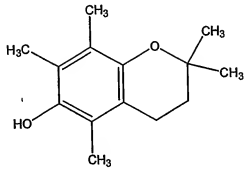
Abbreviations used herein include: AR, androgen receptor; αCEHC, α-carboxyethylhydroxychroman; CSS, charcoal-stripped serum; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MMTV/LTR, Mouse mammary tumor virus long terminal repeat; PBS, phosphate-buffered saline; PMC, 2,2,5,7,8-pentamethylchroman; PMCol, 2,2,5,7,8-pentamethyl-6-chromanol; PSA, prostate-specific antigen; R1881, methyltrienolone.

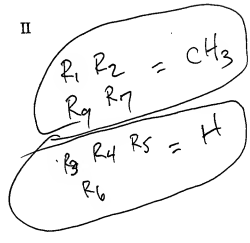
I



wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_9 and R_{10} are independently a substituted or un-substituted C_1-C_3 alkyl group or H; and R_8 is an OH. In a preferred embodiment, the above-described method utilizes an anti-androgen compound having Formula II:

II





In Formula I, the substituent R is defined as an alkyl group, H or OH, unless otherwise indicated. An "alkyl" group refers to a saturated aliphatic hydrocarbon. The alkyl group has 1-3 carbons, and may be un-substituted or substituted by one or more groups selected from halogen, hydroxy, alkoxy carbonyl, amido, alkylamido, dialkylamido, nitro, amino, alkylamino, dialkylamino, carboxyl, thio and thioalkyl.

Substitutions C_{1-3}

embodiment, the compounds are a mixture of the (R) and the (S) isomers. In another embodiment, the compounds are a racemic mixture comprising an equal amount of the (R) and the (S) isomers. It is well known in the art how to prepare optically-active forms (for example, by resolution of the racemic form by recrystallization techniques, by synthesis from optically-active starting materials, by chiral synthesis, or by chromatographic separation using a chiral stationary phase).

The invention includes the use of pharmaceutically acceptable salts of amino-substituted compounds with organic and inorganic acids, for example, citric acid and hydrochloric acid. The invention also includes N-oxides of the amino substituents of the compounds described herein. Pharmaceutically acceptable salts can also he prepared from the phenolic compounds by treatment with inorganic bases, for example, sodium hydroxide. Also, esters of the phenolic compounds can be made with aliphatic and aromatic carboxylic acids, for example, acetic acid and benzoic acid esters. As used herein, the term "pharmaceutically acceptable salt" refers to a compound formulated from a base compound which achieves substantially the same pharmaceutical effect as the base compound.

This invention further includes method utilizing derivatives of the anti-androgen compounds. The term "derivatives" includes but is not limited to ether derivatives, acid derivatives, amide derivatives, ester derivatives and the like. In addition, this invention further includes methods utilizing hydrates of the anti-androgen compounds. The term "hydrate" includes but is not limited to hemihydrate, monohydrate, dihydrate, trihydrate and the like.

This invention further includes methods of utilizing metabolites of the anti-androgen compounds. The term "metabolite" means any substance produced from another substance by metabolism or a metabolic process.

As used herein, receptors for extracellular signaling molecules are collectively referred to as "receptors". Many receptors are transmembrane proteins on a cell surface where they contact or bind an extracellular signaling molecule (i.e., a ligand). In this manner, the receptors initiate a cascade of intracellular signals that alter the behavior of the cell. In contrast, in some cases, the receptors are located within the cell and the signaling ligand must first enter the cell by passive or active transport to activate the receptor.

Steroid hormones are one example of small molecules that diffuse directly across the plasma membrane of target cells and bind to intracellular receptors. These receptors are structurally related and constitute the intracellular receptor superfamily (or steroid-hormone receptor superfamily). Steroid hormone receptors include progesterone receptors, estrogen receptors, androgen receptors, glucocorticoid receptors, and mineralocorticoid receptors. The present invention is particularly directed to androgen receptors. An androgen receptor is an androgen receptor of any species of, for example, a mammal. In one embodiment, the androgen receptor is an androgen receptor of a human.

The invention is directed to methods utilizing anti-androgen compounds which are antagonist compounds. A receptor antagonist is a substance which contacts or interacts with receptors and inactivates them. Thus, an anti-androgen compound useful in the invention binds and inactivates steroidal hormone receptors.

Assays to measure the anti-androgen activity of chroman-derived compounds, as described herein, are well known to a person skilled in the art. For example, androgen receptor antagonistic activity can be determined by monitoring the ability of a candidate anti-androgen compound to inhibit the growth of androgen-dependent tissue, an example of such an assay being provided in the following Example section.

The compounds useful in the present invention bind either reversibly or irreversibly to an androgen receptor. In one embodiment, the anti-androgen compound binds reversibly to an androgen receptor. In another embodiment, the anti-androgen compound binds reversibly to an androgen receptor of a mammal. In another embodiment, the anti-androgen compound binds reversibly to an androgen receptor of a human. Reversible binding of a compound to a receptor means that a compound can dissociate from the receptor after binding.

In another embodiment, the anti-androgen compound binds irreversibly to an androgen receptor. In one embodiment, the anti-androgen compound binds irreversibly to an androgen receptor of a mammal. In another embodiment, the anti-androgen compound binds irreversibly to an androgen receptor of a human. Thus, in one embodiment, the compounds of the present invention may contain a functional group (e.g. affinity label) that allows alkylation of the androgen receptor (i.e. covalent bond formation). In this case, the compounds are alkylating agents which bind irreversibly to the receptor and, accordingly, cannot be displaced by a steroid, such as the endogenous ligands dihydroxy testosterone (DHT) and testosterone. An "alkylating agent" is defined herein as an agent which alkylates (forms a covalent bond) with a cellular component, such as DNA, RNA or protein. For example, in one embodiment, an alkylating group is an isocyanate moiety, an electrophilic group which forms covalent bonds with nucleophilic groups (N, O, S etc.) in cellular components. In another embodiment, an alkylating group is an isothiocyanate moiety, another electrophilic group which forms covalent bonds with nucleophilic groups (N, O, S etc.) in cellular components. In another embodiment, an alkylating group is a haloalkyl (CH_2X wherein X is halogen), an electrophilic group which forms covalent bonds with nucleophilic groups in cellular components. In another embodiment, an alkylating group is a haloalkyl-amido (NH

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II.

As defined herein, "contacting" means that the anti-androgen compound used in the present invention is introduced into a sample containing the receptor in a test tube, flask, tissue culture, chip, array, plate, microplate, capillary, or the like, and incubated at a temperature and time sufficient to permit binding of the anti-androgen compound to the receptor. Methods for contacting the samples with the anti-androgen compound or other specific binding components are known to those skilled in the art and may be selected depending on the type of assay protocol to be run. Incubation methods are also standard and are known to those skilled in the art.

In another embodiment, the term "contacting" means that the anti-androgen compound used in the present invention is introduced into a patient receiving treatment, and the compound is allowed to come in contact with the androgen receptor *in vivo*.

As used herein, the term "treating" includes preventative as well as disorder remittent treatment. As used herein, the terms "reducing", "suppressing" and "inhibiting" have their commonly understood meaning of lessening or decreasing. As used herein, the term "progression" means increasing in scope or severity, advancing, growing or becoming worse. As used herein, the term "recurrence" means the return of a disease after a remission.

As used herein, the term "administering" refers to bringing a patient, tissue, organ or cells in contact with an anti-androgen compound according to Formula I. As used herein, administration can be accomplished *in vitro*, i.e. in a test tube, or *in vivo*, i.e. in cells or tissues of living organisms, for example, humans. In certain embodiments, the present invention encompasses administering the compounds useful in the present invention to a patient or subject. A "patient" or "subject", used equivalently herein, refers to a mammal, preferably a human, that either: (1) has an androgen-dependent

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with a 5-alpha reductase inhibitor. In another embodiment, the present invention provides administering compositions and pharmaceutical compositions comprising an anti-androgen compound, in combination with an aromatase inhibitor. In another embodiment, the present invention provides administering compositions and pharmaceutical compositions comprising an anti-androgen compound, in combination with a progestin. In another embodiment, the present invention provides administering compositions and pharmaceutical compositions comprising an anti-androgen compound, in combination with an agent acting through other nuclear hormone receptors.

As used herein, "pharmaceutical composition" means therapeutically effective amounts of the anti-androgen compound together with suitable diluents, preservatives, solubilizers, emulsifiers, and adjuvants, collectively "pharmaceutically-acceptable carriers." As used herein, the terms "effective amount" and "therapeutically effective amount" refer to the quantity of active therapeutic agent sufficient to yield a desired therapeutic response without undue adverse side effects such as toxicity, irritation, or allergic response. The specific "effective amount" will, obviously, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives. In this case, an amount would be deemed therapeutically effective if it resulted in one or more of the following: (a) the prevention of an androgen-mediated disorder (e.g., prostate cancer); and (b) the reversal or stabilization of an androgen-mediated disorder (e.g., prostate cancer). The optimum effective amounts can be readily determined by one of ordinary skill in the art using routine experimentation.

Pharmaceutical compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl, acetate,

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Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

Controlled or sustained release compositions administerable according to the invention include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

Other embodiments of the compositions administered according to the invention incorporate particulate forms, protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the

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compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

In yet another method according to the invention, a pharmaceutical composition can be delivered in a controlled release system. For example, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, i.e., the prostate, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984). Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990).

The pharmaceutical preparation can comprise the anti-androgen compound alone, or can further include a pharmaceutically acceptable carrier, and can be in solid or liquid form such as tablets, powders, capsules, pellets, solutions, suspensions, elixirs, emulsions, gels, creams, or suppositories, including rectal and urethral suppositories. Pharmaceutically acceptable carriers include gums, starches, sugars, cellulosic materials, and mixtures thereof. The pharmaceutical preparation containing the anti-androgen compound can be administered to a patient by, for example, subcutaneous implantation of a pellet. In a further embodiment, a pellet provides for controlled release of anti-androgen compound over a period of time. The preparation can also be administered by

intravenous, intraarterial, or intramuscular injection of a liquid preparation oral administration of a liquid or solid preparation, or by topical application. Administration can also be accomplished by use of a rectal suppository or a urethral suppository.

The pharmaceutical preparations administerable by the invention can be prepared by known dissolving, mixing, granulating, or tablet-forming processes. For oral administration, the anti-androgens or their physiologically tolerated derivatives such as salts, esters, N-oxides, and the like are mixed with additives customary for this purpose, such as vehicles, stabilizers, or inert diluents, and converted by customary methods into suitable forms for administration, such as tablets, coated tablets, hard or soft gelatin capsules, aqueous, alcoholic or oily solutions. Examples of suitable inert vehicles are conventional tablet bases such as lactose, sucrose, or cornstarch in combination with binders such as acacia, cornstarch, gelatin, with disintegrating agents such as cornstarch, potato starch, alginic acid, or with a lubricant such as stearic acid or magnesium stearate.

Examples of suitable oily vehicles or solvents are vegetable or animal oils such as sunflower oil or fish-liver oil. Preparations can be effected both as dry and as wet granules. For parenteral administration (subcutaneous, intravenous, intraarterial, or intramuscular injection), the anti-androgen compounds or their physiologically tolerated derivatives such as salts, esters, N-oxides, and the like are converted into a solution, suspension, or expulsion, if desired with the substances customary and suitable for this purpose, for example, solubilizers or other auxiliaries. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene

solutions, suspensions, or emulsions in a physiologically acceptable diluent with or without a pharmaceutical carrier.

In another method according to the invention, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, N.Y., pp. 353-365 (1989); Lopez-Berestein ibid., pp. 317-327; see generally ibid).

For use in medicine, the salts of the anti-androgen compound may be pharmaceutically acceptable salts. Other salts may, however, be useful in the preparation of the compounds according to the invention or of their pharmaceutically acceptable salts. Suitable pharmaceutically acceptable salts of the compounds include acid addition salts which may, for example, be formed by mixing a solution of the compound according to the invention with a solution of a pharmaceutically acceptable acid such as hydrochloric acid, sulphuric acid, methanesulphonic acid, fumaric acid, maleic acid, succinic acid, acetic acid, benzoic acid, oxalic acid, citric acid, tartaric acid, carbonic acid or phosphoric acid.

In addition, the anti-androgen compounds described herein may be provided in the form of nutraceutical compositions where the anti-androgen compound prevents the onset of or reduces or stabilizes various deleterious androgen-related disorders, e.g., prostate cancer. The term "nutraceutical," or "nutraceutical composition", for the purposes of this specification, refers to a food item, or a part of a food item, that offers medical health benefits, including prevention and/or treatment of disease. A nutraceutical composition according to the present invention may contain only an anti-androgen compound according to the present invention as an active ingredient or, alternatively, may further comprise, in admixture with the aforesaid anti-androgen

compound, dietary supplements including vitamins, co-enzymes, minerals. herbs, amino acids and the like which supplement the diet by increasing the total intake of that substance.

Therefore, the present invention provides methods of providing nutraceutical benefits to a patient comprising the step of administering to the patient a nutraceutical composition containing a compound having Formula I or a pharmaceutically acceptable salt thereof. Such compositions generally include a "nutraceutically-acceptable carrier" which, as referred to herein, is any carrier suitable for oral delivery including, but not limited to, the aforementioned pharmaceutically-acceptable carriers. In certain embodiments, nutraceutical compositions according to the invention comprise dietary supplements which, defined on a functional basis, include immune boosting agents, anti-inflammatory agents, anti-oxidant agents, or mixtures thereof.

The immune boosters and/or anti-viral agents are useful for accelerating wound-healing and improved immune function; and they include extracts from the coneflowers, or herbs of the genus *Echinacea*, extracts from herbs of the genus *Sambuca*, and Goldenseal extracts. Herbs of the genus *Astragalus* are also effective immune boosters in either their natural or processed forms. *Astragalus* stimulates development into of stem cells in the marrow and lymph tissue active immune cells. Zinc and its bioactive salts, such as zinc gluconate and zinc acetate, also act as immune boosters in the treatment of the common cold.

Antioxidants include the natural, sulfur-containing amino acid allicin, which acts to increase the level of antioxidant enzymes in the blood. Herbs or herbal extracts, such as garlic, which contain allicin are also effective antioxidants. The catechins, and the extracts of herbs such as green tea containing catechins, are also effective antioxidants.

Extracts of the genus *Astragalus* also show antioxidant activity. The bioflavonoids, such as quercetin, hesperidin, rutin, and mixtures thereof, are also effective as antioxidants. The primary beneficial role of the bioflavonoids may be in protecting vitamin C from oxidation in the body. This makes more vitamin C, or ascorbic acid, available for use by the body.

Bioflavonoids such as quercetin are also effective anti-inflammatory agents, and may be used as such in the inventive compositions. Anti-inflammatory herbal supplements and anti-inflammatory compounds derived from plants or herbs may also be used as anti-inflammatory agents in the inventive composition. These include bromolain, a proteolytic enzyme found in pineapple; teas and extracts of stinging nettle; turmeric, extracts of turmeric, or curcumin, a yellow pigment isolated from turmeric.

Another supplement which may be used in the present invention is ginger, derived from herbs of the genus *Zingiber*. This has been found to possess cardiotonic activity due to compounds such as gingerol and the related compound shogaol as well as providing benefits in the treatment of dizziness, and vestibular disorders. Ginger is also effective in the treatment of nausea and other stomach disorders.

Supplements which assist in rebuilding soft tissue structures, particularly in rebuilding cartilage, are useful in compositions for treating the pain of arthritis and other joint disorders. Glucosamine, glucosamine sulfate, chondroitin, and chondroitin sulfate are particularly useful for this purpose. Chondroitin may be derived from a variety of sources, such as Elk Velvet Antler. Marine lipid complexes, omega 3 fatty acid complexes, and fish oil are also known to be useful in treating pain associated with arthritis.

Extracts of the genus *Astragalus* also show antioxidant activity. The bioflavonoids, such as quercetin, hesperidin, rutin, and mixtures thereof, are also effective as antioxidants. The primary beneficial role of the bioflavonoids may be in protecting vitamin C from oxidation in the body. This makes more vitamin C, or ascorbic acid, available for use by the body.

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B. BACKGROUND

The activity of androgens is tissue-specific and mediated through the androgen receptor (AR). The disruption of androgens and AR activity alters the regulation of androgen-sensitive tissues, such as the prostate gland (1). In the prostate, androgens have a central role in normal glandular development and function (2). However, androgens are also necessary for the development of prostate cancer. The role of androgens in prostate cancer development is emphasized by the observation that eunuchs and men that have a mutation in 5α-reductase type II, an enzyme that converts testosterone to the more potent dihydrotestosterone, do not develop prostate cancer (3). The incidence of prostate cancer has continued to rise for the last two decades, currently affecting over 200,000 men in the United States each year (4). Agents that permit the necessary actions of androgen for normal tissue function while reducing the role of androgens in the pathogenesis of androgen-sensitive tissues may serve as a useful means of reducing prostate cancer development. Recently, several agents have been reported to prevent prostate cancer development, such as selenium, lycopene, and vitamin E (5).

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cells (13). The exquisite sensitivity of LNCaP cells to androgenic stimulation may be due to a mutation in the ligand-binding domain of the androgen receptor (14). To date, the LNCaP prostate cell line has been the most extensively characterized prostate cell line for examining the effects of androgens. More recently, the LAPC-4 cell line has been introduced as another androgen-sensitive human prostate carcinoma cell line that expresses a normal AR (15). However, the response of LAPC-4 cells to androgens is not as pronounced as observed in LNCaP cells. Collectively, the LNCaP and LAPC4 human prostate carcinoma cell lines provide valuable models for investigating androgen-regulated cellular pathways.

Previous studies have focused primarily on the inhibition of prostate cell growth by vitamin E treatment, which may occur through effects on cell cycle regulators (16, 17, 18). Apoptotic responses induced by vitamin E treatment have also been observed in LNCaP cells (19, 20). Interestingly, vitamin E-induced apoptotic responses were enhanced by coadministration of androgen (19). Zhang et al (21) reported that vitamin E succinate reduces the levels of the AR in LNCaP cells, with resultant inhibition of androgen-mediated responses. However, the direct actions of vitamin E and related compounds on androgen receptor activity in prostate cells have not been extensively examined. In the study described below, the androgen receptor antagonist activity and modulation of androgen-sensitive pathways by the vitamin E derivative, PMCol, were investigated by the present inventors in human prostate carcinoma cells.

C. MATERIALS AND METHODS

Chemicals. PMCol and PMC were obtained from Aldrich (Milwaukee, WI). The chemical structures of α-tocopherol, PMCol, and PMC are shown in Figure 1. Bicalutamide (Casodex) was kindly provided by AstraZeneca Pharmaceuticals

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Cell growth and viability analyses. Five thousand LNCaP or LAPC4 cells were plated in each well of 96-well plates (Costar) in 100 µl of DMEM/CSS. Two to 3 d after plating, cells were treated by adding 100 µl of DMEM/CSS containing 2 × the concentration of the specified treatment to each well. Four d after treatment, the relative cell number was estimated by the determining DNA concentration of each well using a Hoechst-based fluorescence DNA assay, as previously described (23). Growth analysis with DU145 cells was performed similar to those with LNCaP and LAPC4 cells except DU145 cells were initially seeded at 500 cells per well. Cell viability was determined by trypan blue exclusion and quantified by light microscopic analysis using a hemacytometer.

Determination of secreted PSA levels. LNCaP cells were cultured in 96 well plates (Costar) at 5,000 cells per well in DMEM/CSS 1 d before treatment. Forty-eight h after treatment, PSA levels in cell culture media were determined using the Tandem-MP PSA kit (Beckman Coulter, Inc.) according to manufacturer's instructions. PSA levels were normalized to DNA levels as determined using a Hoechst-based fluorescence DNA assay (23).

Androgen–stimulated promoter reporter assay analysis. LNCaP and LAPC4 prostate carcinoma cell lines were cultured in 12-well cell culture plates (Costar) in DMEM/CSS 2 to 3 d before transfection. Androgen-induced trancriptional activation was determined using a reporter construct with an MMTV promoter that regulates the expression of luciferase (24). LNCaP and LAPC4 cells were transfected with the MMTV/luciferase plasmid using the Effectene Transfection Reagent (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Twenty-four h after transfection, cells were treated with R1881 with or without test reagents at the specified

concentrations. Cell extracts were acquired 24 to 48 h after treatment by removing medium, washing 1 × with PBS, and obtaining extract with 200 µL of 1× Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was determined as previously described (24).

Immunoblot analysis of AR protein levels. LNCaP cells were plated at a density of 1×10^6 cells per 100 mm cell culture plate in 10 ml of DMEM/FBS and maintained in incubators at 37°C in 5% CO_2. After 5 d of treatment with vehicle, 30 µM PMC, 30 µM PMCol, or 1.0 µM bicalutamide, cells were washed in cold 1× PBS and lysed in a buffer containing 1.0 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 µg/ml aprotinin in 1× PBS. Total protein (10 µg) from cell extracts were electrophoresed on 7.5 % SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a GENIE wet transfer system (Idea Scientific, Minneapolis, MN). Membranes were blocked in Tris-buffered saline containing 5% nonfat dry milk at and then incubated with mouse anti-AR (441) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-actin antibody (A5441; Sigma). Membranes were then incubated with a secondary horseradish peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using the Enhanced Chemiluminescence Plus reagent (Amersham Pharmacia Biotech). Autoradiograms were prepared by exposing the blots to BioMax Light X-ray film (Eastman Kodak Co., Rochester, NY) and developed using a CURIX 60 CP Processor (Agfa, Ridgefield Park, NJ).

Statistical analysis. Significant differences in values between groups were assessed using a two-sided Student's T-test. *P* values less than 0.05 were used to signify statistical significance.

D. RESULTS

PMCol Inhibits Androgen Binding in Prostate Cancer Cells. AR competition was determined using ^{3}H-R1881 in the androgen-sensitive LNCaP cell line, which expresses a functional mutant AR (25), and the LAPC4 cell line, which express a normal human AR (15). Increasing concentrations of the AR antagonist bicalutamide were found to progressively inhibit R1881 binding (Fig. 2A), with an estimated IC_{50} of 0.7 μM in LNCaP cells. PMCol was found to be approximately 10-fold less potent at competing for ^{3}H-R1881 than bicalutamide in LNCaP cells, with an estimated IC_{50} of 7.2 μM (Fig. 2A). Repeated studies of PMCol competition for ^{3}H-R1881 binding gave IC_{50} values ranging from 5 to 15 μM (data not shown). In contrast, PMC, in which the 6-hydroxyl of PMCol is absent, had less anti-androgenic activity than PMCol (Fig. 2A) and significantly reduced cell viability at a concentration of 100 μM within 2 h of treatment (data not shown). Based on the R1881 competition results in LNCaP cells (Fig.2A), a dose of 30 μM PMC and PMCol was used in most of these studies, allowing an effective comparison of the anti-androgenic activity between PMC and PMCol. In LAPC4 cells, treatment with 30 μM PMCol produced a 75% decrease in ^{3}H-R1881 binding and treatment with 1 μM bicalutamide produced a 62% decrease in ^{3}H-R1881 binding (Fig. 2B).

Modulation of prostate carcinoma cell growth and viability by PMCol. Changes in growth of the androgen-independent DU145 prostate carcinoma cell line and the androgen-sensitive LNCaP and LAPC4 prostate cell lines were assessed at

concentrations of PMCol ranging from 10 to 100 μM (Fig. 3A). Concentrations of 50 μM, 60 μM, and 80 μM or more PMCol were required to significantly reduce cell growth in LNCaP, LAPC4, and DU145 cells, respectively (Fig. 3A). LNCaP cells produce a biphasic growth response to androgen exposure (9). Modulation of LNCaP cell growth by PMCol treatment was examined over 4 d. PMCol had no growth modulatory activity in vehicle-control treated LNCaP cells grown in androgen-deficient media (i.e., PMCol did not have AR agonist activity) at concentrations ranging from 10 μM to 30 μM PMCol (Fig. 3B). However, LNCaP cell growth was decreased at concentrations equal to or higher than 40 μM PMCol (Fig. 3B) and PMCol concentrations of 100 μM or greater produced significant cell death at 48 and 96 h (Table I). Stimulation of LNCaP growth by exposure to 0.1 nM R1881 was significantly inhibited by treatment with concentrations of 10 μM or more PMCol (Fig. 3B). However, a significant stimulation in LNCaP cell growth was observed in the presence of a normally growth inhibitory concentration of 1.0 nM R1881 with treatment of 10 μM to 30 μM PMCol (Fig. 3B). The R1881-stimulated growth curve of LNCaP cells was shifted to the right in the presence of 30 μM PMCol, similar to that produced by treatment with 1 μM bicalutamide (Fig. 4). A more modest, but significant, shift to the right in the androgen-induced LNCaP growth curve was observed by treatment with 30 μM PMC (Fig. 4).

Table 1 Time- and dose-dependent changes in LNCaP cell viability after PMCol treatment

	% Cell Viability[a] (SD)					
	[PMCol] (μM)					
Time (h)	0	25	50	75	100	250
48	92.3 (4.7)	90.0 (2.8)	88.0 (3.4)	80.0 (12.5)	71.0 (8.7)[b]	11.0 (8.6)[b]
96	88.0 (2.5)	87.0 (4.8)	85.0 (4.6)	87.0 (4.0)	21.0 (3.3)[b]	2.0 (1.8)[b]

[a] Determined by trypan blue exclusion analysis and quantified using a hemacytometer.

[b] Significantly different compared to 0 μM PMCol ($P<0.05$; $n=4$).

Inhibition of PSA secretion by PMCol in LNCaP cells. PSA secretion by LNCaP cells is stimulated by androgen exposure in a dose-dependent manner (12). The R1881-stimulated production of PSA from LNCaP cells was measured after PMCol treatment for 48 h. PSA release from LNCaP cells was not affected by treatment with 30 μM PMCol alone (Fig. 5). However, PSA levels were increased 3.1-fold after exposure to a growth stimulatory dose of 0.05 nM R1881, which was completely inhibited by treatment with 30 μM PMCol (Fig. 5). Exposure of LNCaP cells to 1.0 nM R1881 produced a 12-fold increase in PSA levels by 48 h, which was decreased 20%, 81%, and 43% by treatment with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide, respectively (Fig. 5).

Inhibition of androgen-stimulated transcriptional activation by PMCol. Studies on androgen-regulated transcriptional activation were performed in LNCaP and LAPC4 cells transiently transfected with a reporter vector that uses the androgen-sensitive MMTV/LTR to drive expression of a luciferase reporter gene. In LNCaP cells, PMCol

treatment alone had no effect on MMTV promoter activity, whereas luciferase expression was increased 54-fold after exposure to 1.0 nM R1881 for 24 h (Fig. 6A). Luciferase expression induced by exposure to 1.0 nM R1881 in LNCaP cells for 24 h was decreased 50% and 70% by treatment with 25 μM and 50 μM PMCol, respectively (Fig. 6A). Similarly, LAPC4 cells exposed to 1.0 nM R1881 produced a 20-fold increase in MMTV/LTR driven luciferase expression that was decreased 60% by treatment with 30 μM PMCol after 24 h (Fig. 6B). In both LNCaP and LAPC4 cells, treatment with 1 μM bicalutamide decreased 1.0 nM R1881-stimulated luciferase expression approximately 50% (Fig. 6A and 6B).

Androgen receptor protein levels in PMCol exposed LNCaP cells. Previous studies in LNCaP cells have reported that AR levels are decreased after treatment with vitamin E analogs, which may account for the reduced sensitivity of these cells to androgen exposure (21). However, in the current study, LNCaP cells treated with 30 μM PMC, 30 μM PMCol, or 1 μM bicalutamide for 5 d did not result in altered AR protein levels (Fig. 7).

E. DISCUSSION

In the current study, the inventors examined the effects of an agent traditionally considered as an antioxidant on prostate carcinoma cells. Epidemiological studies provide intriguing evidence that antioxidant dietary factors such as β-lycopene and vitamin E may help prevent prostate cancer development (5). Although these agents have been classified as antioxidants, the mechanism by which they may contribute to prostate cancer prevention has not been firmly established. Androgens are known to have an essential role in prostate cancer development (3). Modulation of androgen activity may provide a means of prostate cancer prevention (26). Here, the inventors

have determined the antioxidant moiety of vitamin E, PMCol, to be a potent anti-androgen in androgen-sensitive human prostate carcinoma cells.

The LNCaP human prostate carcinoma cell line is one of the few prostate cell lines that show demonstrable physiologic changes resulting from androgen exposure, such as growth modulation (9). Therefore, the LNCaP cell line has proven valuable in identifying agents that alter androgen-stimulated cell growth. In the current study, PMCol shifted the androgen-mediated growth curve in LNCaP cells such that higher androgen concentrations were necessary to produce the biphasic growth response typically observed in LNCaP cells. The LNCaP growth shift with PMCol treatment was sufficient to produce growth stimulation in the presence of 1.0 nM R1881, a concentration of R1881 that typically inhibits LNCaP proliferation (10). The shift in LNCaP growth pattern observed with PMCol treatment was similar to that observed in LNCaP cells after treatment with the pure anti-androgen bicalutamide. Also, the IC_{50} of PMCol observed in an androgen competition analysis for R1881 binding in LNCaP cells is in agreement with the dose-response shift in androgen-mediated growth of LNCaP cells after PMCol treatment. Together, these results suggest that the shift observed in the androgen-mediated growth of LNCaP cells was due to the anti-androgenic activity of PMCol.

Although LNCaP cells have proven to be useful in evaluating androgen-responsive pathways, the use of LNCaP cells to assess anti-androgenic activity can be inaccurate since LNCaP cells harbor a mutant AR (25). The AR receptor in LNCaP cells, which although functional, has been reported to have altered ligand binding affinity (14) and is stimulated by some agents that are antagonists for the wild-type AR (22). Therefore, in this study, competition for AR binding by PMCol was also assessed in the

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LAPC4 human prostate carcinoma cell line, which expresses a wild-type AR (15). PMCol competition for R1881 binding was found to be similar for LNCaP and LAPC4 cells. In addition, the pure anti-androgen bicalutamide was found to have equivalent AR competition activity in LNCaP and LAPC4 cells. Therefore, the pure anti-androgen bicalutamide and PMCol were found to possess comparable AR antagonist activity in LNCaP cells, expressing a functional mutant AR, and LAPC4 cells, which express a normal AR.

The AR functions primarily as a transcription factor that is activated by androgen binding (1). In these studies, the androgen-responsive MMTV promoter was used to assess modulation of androgen-stimulated transcriptional activity. Upon androgen exposure (i.e., R1881), MMTV promoter activity was stimulated in both LNCaP and LAPC4 cells. Also, in both cell lines, R1881-stimulation of MMTV activity was significantly inhibited by PMCol treatment. PMCol treatment alone did not stimulate MMTV promoter activity (i.e., PMCol was not found to have AR agonist or partial agonist activity). The effects of androgen exposure on transcriptional activation were further observed by the inhibition of androgen-stimulated PSA release after treatment with PMCol in LNCaP cells. Previously, vitamin E succinate was reported to inhibit the effects of androgen on LNCaP cells through down-regulation of androgen receptor levels (21). Other agents, such as curcumin, have been shown to decrease AR expression in LNCaP cells (27). In the current study, LNCaP cells treated with 30 μM PMCol for five days did not affect AR protein levels. Then, PMCol was found to be a potent inhibitor of transcriptional activation of androgen-responsive promoters, likely through directly blocking AR activation by androgen.

In the current study, PMC, which lacks the phenolic hydroxyl group present on PMCol, was less potent than PMCol at inhibiting androgenic responses. Therefore, the phenolic hydroxyl group of the chromanol ring contributes significantly to the anti-androgenic activity of PMCol. Other forms of vitamin E, such as β-, γ-, and δ-tocopherol differ from α-tocopherol by the number and location of methyl group substitutions on the chromanol ring (7). The inventors can propose that the antioxidant moieties of other forms of vitamin E also possess anti-androgenic activity with potencies that vary dependent on the specific methyl group substitutions present on the chromanol ring.

A variety of dietary agents have been identified that have anti-androgenic activity in prostate carcinoma cells. However, the mechanism of anti-androgenic activity observed by dietary anti-androgens may vary. For example, curcumin, a component of turmeric, was reported to down-regulate androgen receptor protein levels in LNCaP cells, which effectively attenuates androgenic responses (27). In contrast, indole-3-carbinol, a component of cruciferous vegetables, when converted to diindolylmethane was reported to act as a potent inhibitor of androgen binding in LNCaP cells, but does not affect AR protein levels (28). Zhang et al. (21), have reported that vitamin E succinate is inhibitory to androgenic responses in LNCaP cells through down-regulation of AR protein levels, similar to the action of curcumin. By contrast, in the current study, the inventors found that the antioxidant moiety of vitamin E, PMCol, effectively blocks androgen binding to the AR without affecting AR protein levels, similar to effects observed with indole-3-carbinol derivatives (28). Therefore, dietary anti-androgens may serve as an effective means of modulating androgenic pathways through a variety of mechanisms affecting AR activity.

PMCol has largely been investigated for its antioxidant activity associated with being the antioxidant moiety of vitamin E. For example, the antioxidant potency of PMCol was shown to be similar to α-tocopherol *in vitro* (29). In general, α-tocopherol plasma levels range between 5 and 30 μM (30), well within the range of anti-androgenic activity observed by PMCol in the current study. Due to the high lipophilicity of vitamin E, it is difficult to assess its anti-androgenic activity by cell culture analysis. However, due to the presence of the highly lipophilic phytyl chain, the subcellular distribution of vitamin E would limit its direct interaction with the AR, which resides in more aqueous subcellular compartments such as the cytoplasm and nucleus Vitamin E can be metabolized to derivatives with greater water solubility, such as αCEHC (7, 31), which are structurally similar to PMCol, and may have greater water solubility and a distinct cellular bioavailability compared to vitamin E. Thus, metabolites of vitamin E may contact the AR *in vivo* and have anti-androgenic activity, analogous to that produced by PMCol in human prostate carcinoma cells.

In summary, the antioxidant moiety of α-tocopherol, PMCol, was found by the present inventors to inhibit androgen activity, likely through competing for androgen binding to the AR, with resultant inhibition of androgen-sensitive biological pathways. PMCol was not found to possess androgen agonist or partial agonist activity and hence functions as a pure antagonist of androgen activity in the LNCaP and LAPC4 prostate carcinoma cell lines. Based on the results of the current study, PMCol will serve as a useful agent for modulating androgen activity *in vivo*. Importantly, the anti-androgenic activity of PMCol poses the possibility that the prostate cancer preventive activity of vitamin E may, in part, be due to anti-androgenic effects of vitamin E or metabolites of vitamin E in the prostate. Currently, over 30,000 men die from prostate cancer each year in the United States (4). The prevention of prostate cancer through the action of PMCol

and derivatives thereof, offers an effective means of reducing the devastation produced by this disease.

Example 2. Acute Oral Toxicity in Mice

The oral toxicity of PMCol was determined in 6 month-old male FVB mice. A single high oral dose of 1000 mg/kg PMCol in sesame oil was administered to 4 mice by gavage. Four control mice received only sesame oil by gavage (vehicle control). No significant change in animal behavior or body mass (Fig. 8A) occurred after administration of PMCol or vehicle control for up to 1 week after PMCol administration. In a second study, four 6 month-old male FVB mice received 200 mg/kg PMCol daily in sesame oil by gavage for 10 days. Three mice receiving only sesame oil (vehicle control) were used as controls. Body weights were determined daily and all mice were autopsied to examine gross organ changes on day 11. No significant difference in body mass change was observed in comparing PMCol-treated and vehicle control mice over 10 days (Fig. 8B). No gross changes in organs were observed for either PMCol-treated or control mice. For example, liver mass was not significantly changed in mice receiving PMCol for 10 days (Fig. 8C). Therefore, the LD50 of PMCol in mice is greater than the highest dose tested (i.e., 1000 mg/kg body weight) and PMCol is well tolerated in mice at high doses for up to 10 days.

Example 3. Determining *in vivo* efficacy of a chroman-derived anti-androgen using the LNCaP xenograft model and the TRAMP prostate carcinogenesis model.

A nude mouse/LNCaP xenograft model, similar to the DU145 xenograft method previously described (Church *et al.*, Cancer Chemother. Pharmacol. 43:198-204 (1999)), may be used to examine the *in vivo* actions of PMCol on human prostate carcinoma cell growth. Male Hsd: athymic nude-*nu* (*nu/nu*, BALB/c origin) mice at 4 weeks of age will

be acquired from Harlan Sprague Dawley (Madison, WI). At 6 weeks of age, each mouse will be subcutaneously xenografted with 10^6 LNCaP cells in 0.1 mL of medium + 0.1 mL of Matrigel (BD Biosciences) in flanking ventral fat pads. One week after LNCaP xenografting, mice will be divided into 5 treatment groups of 10 mice each. Mice in group 1 will receive a vehicle control of 0.25 mL of corn oil by gavage, group 2 will receive 25mg/kg of flutamide (Sigma Chemical Co., St. Louis, MO) as an anti-androgen treatment control, group 3 will receive 25 mg/kg of PMCol in 0.25 mL corn oil, and group 4 will receive 100 mg/kg of PMCol in 0.25 mL corn oil. Dosages are based on toxicity studies described above. Group 5 will be castrated 1 week after LNCaP xenografting as a low androgen control. Each mouse will be treated daily for 2 months. LNCaP tumor growth will be determined twice weekly and tumor volume will be determined. Two months after LNCaP xenografting, mice will be sacrificed and all LNCaP tumors will be removed and fixed in 10 % formalin for histological examination by light microscopy. At the time of euthanasia, blood will be collected to determine circulating testosterone, luteinizing hormone, and PMCol levels as performed below. Also, livers and male sexual accessory organs (i.e., seminal vesicles and prostate lobes) will be collected from each mouse for analysis of PMCol's effects on these tissues.

The TRAMP prostate carcinogenesis model will be used to assess the anti-androgenic activity of PMCol on androgen-dependent tumor growth in the mouse prostate using a TRAMP mouse colony maintained on a C57BL/6 background. At 3-months of age, before the onset of prostate carcinogenesis, heterozygous male TRAMP mice will be divided into 5 treatment groups, as described above. Flutamide's efficacy in the TRAMP model has been reported (Raghow *et al.*, Cancer Res. 60: 4093-4097 (2000)). TRAMP mice on study will be treated daily. Four months after the initiation of treatment, at which point approximately 50% of the mice show demonstrable prostatic

adenocarcinomas, mice will be sacrificed and the prostate lobes and sex accessory glands will be removed and fixed in 10% formalin and prepared for histological analysis. Hematoxylin and eosin stained slides of prostate glands will be examined for the presence of prostatic adenocarcinomas, which will be quantified for each treatment group and used to determine the incidence of prostate carcinomas in control versus treatment groups. Blood will also be collected to determine circulating testosterone, luteinizing hormone, and PMCol levels as performed below.

Determining the effect of the vitamin E analog PMCol on central nervous system feedback control of testosterone and luteinizing hormone blood levels compared to PMCol blood levels will be performed as follows. Four-month-old male ICR mice (Harlan Sprague Dawley) will be used to assay the effect of PMCol administration on blood testosterone levels. Mice will be divided into 5 groups of 5 mice each. Mice in group 1 will receive a vehicle control of 0.25 mL of corn oil by gavage, group 2 will receive 25 mg/kg of flutamide (Sigma) as a treatment control antiandrogen, group 3 will receive 25 mg/kg of PMCol in 0.25 mL corn oil, and group 4 will receive 100 mg/kg of PMCol in 0.25 mL corn oil. Group 5 will be castrated as a low androgen control. Mice will be treated daily for 1 month and blood samples will be collected twice a week by retro-orbital bleed, as previously performed (Church *et al.*, 1999). Blood testosterone levels will be determined using a Testosterone EIA Test Kit (BioCheck, Inc., Burlingame, CA). In addition, the testosterone blood levels determined by the EIA kit will be validated using LC-MS. The luteinizing hormone levels in the blood samples will be determined using the Luteinizing Hormone EIA Test Kit (BioCheck, Inc., Burlingame, CA) according to kit instructions. Finally, PMCol blood levels will be determined from the samples using LC-MS.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

IV. REFERENCES

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